Glycosylation of CD44 Negatively Regulates Its Recognition of Hyaluronan

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Summary

Although CD44 is expressed on a wide variety of cell types, few of them use it to recognize the ligand hyaluronan (HA). A glycosylation-defective clone of Chinese hamster ovary cells (Lec 8) bound HA, demonstrating that complete processing of glycoproteins with addition of a full complement of sialic acid is not required. On the contrary, subsequent findings revealed that complex sugars on CD44 can actually inhibit ligand recognition. Two subclones of wild-type Chinese hamster ovary cells with similar amounts of surface CD44 were isolated on the basis of HA binding and found to differ with respect to CD44 size as well as staining with fluorescent lectins. Treatment of the nonbinding clone with tunicamycin reduced the size of the protein and allowed the cells to recognize HA via CD44. This function was also induced by treatment with deglycosylating enzymes (either a mixture of endoglycosidase F and *N*-glycosidase F or neuraminidase alone). A possible role for glycosylation in regulation of adhesion was then sought with a series of normal and transformed murine cells. Disruption of glycosylation or treatment with deglycosylating enzymes did not induce ligand binding in an interleukin 7–dependent pre–B cell line, and splenic B cells also appeared to be in an inactive state. Some normal B cells acquired the ability to recognize HA after stimulation with lipopolysaccharide or interleukin 5 and had distinctive surface characteristics (loss of immunoglobulin D and acquisition of CD43). An additional subset of activated cells might have been in a transitional state, because the cells bound ligand after neuraminidase treatment. The ligand-binding ability of a purified CD44–immunoglobulin fusion protein dramatically increased after neuraminidase treatment. Thus, differential glycosylation of this molecule is sufficient to influence its recognition function. Cell adhesion involving HA can be regulated by multiple mechanisms, one of which involves variable glycosylation of CD44.

The adhesiveness of immature blood cells must be modulated as they leave the bone marrow and controlled thereafter as they interact with vessel walls, cells of the immune system, and the extracellular matrix (1). While the density of CD44 changes on cells in bone marrow, it is more dramatically expressed, repressed, and reexpressed as T lymphocytes mature in the thymus (for review see reference 2). Levels of CD44 tend to increase with activation of B and T lymphocytes, and CD44 can be shed actively into the circulation by lymphoid and myeloid cells (3, 4). In addition to changes in expression and density, other mechanisms may regulate the function of this cell adhesion molecule (for review see reference 2). Although most blood cells are CD44 positive, only small numbers use CD44 to recognize hyaluronan (HA).[1] While resting B cells do not bind HA, activated B lymphoblasts/plasmacytes do so with high affinity. Resting T cells and some cell lines represent an intermediate state, in which HA recognition is minimal but can be dramatically augmented by the CD44 mAb IRAWB14. Days are required after stimulation for resting B lymphocytes with inactive CD44 to express avid HA receptors (5). The slow kinetics of change contrasts with many well-studied integrins, whose specificity and avidity for ligands can be rapidly altered (6).

The function of CD44 might be regulated by interaction with cytoplasmic or surface molecules in the same cell or via ligands on opposing cells. This could result in oligomerization of the receptor into high density domains and/or cause a change in its conformation. While the amino-terminal portion of CD44 binds ligand, both transmembrane and cytoplasmic segments are important for determining whether this happens on intact cells (7, 8). Furthermore, there is evidence that kinases, lipids, and cytoskeletal components associate with CD44 in ways that might alter ligand recognition (9–13). Artificial multimerization of CD44 achieved by replacement of the transmembrane segment increased its ability to bind HA, and the IRAWB14 mAb was ineffective as a monomeric

[1] *Abbreviations used in this paper:* CHO, Chinese hamster ovary; endo-F, endoglycosidase F; FL-HA, fluorescein-conjugated HA; HA, hyaluronan; MAA, *Maacia amurensis*; N'ase, neuraminidase; NZB, New Zealand black; NZW, New Zealand white; PNGase-F, *N*-glycosidase F; RCA, *Ricinus communis*; SNA, *Sambucus nigra*; UEA1, *Ulex europaeus*; WGA, wheat germ agglutinin.

HA, and the IRAWB14 mAb was ineffective as a monomeric Fab fragment (8, 14). However, recent analysis of the IRAWB14 epitope indicates that, in addition to receptor clustering, this antibody influences ligand binding by other means (14a).

Structural heterogeneity in the CD44 protein might also account for variations in its ability to recognize HA. Alternative splicing of particular exons can result in an inactive molecule (15). However, this may depend on the type of cell that expresses it, because the same variant of CD44 is active in other situations (16, 17). It is clear that lymphocytes that express similar CD44 isoforms can differ with respect to HA recognition (2, 18). We now describe another mechanism through which HA binding by CD44 can be regulated. Treatments that disrupt protein glycosylation lead to increased HA recognition in some cell types. Any number of molecules might have been altered in those circumstances. However, neuraminidase (N'ase) treatment of a purified CD44–Ig fusion protein resulted in enhanced ability to bind HA. We conclude that carbohydrate modification of CD44 limits its ability to recognize an abundant natural ligand. These findings could increase understanding of the role of CD44 in processes such as tumor metastasis, in which HA recognition appears to be critical (19). They also extend studies demonstrating that glycoconjugates are important regulators of cell adhesion mediated by other classes of molecules (20–24).

Materials and Methods

Animals. BALB/c and New Zealand black (NZB) × New Zealand white (NZW) F_1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).

Cells, Cultures, and Antibodies. Wild-type Chinese hamster ovary (CHO) cells and Lec 8 CHO cells (25) were kindly provided by Dr. Richard Cummings (Department of Biochemistry and Molecular Biology, University of Oklahoma Health Science Center, Oklahoma City, OK). CHO/SKN and CHO/SKP were subcloned from wild-type CHO cells on the basis of HA recognition. They were all cultured in MEM supplemented with 50 μM 2-ME, L-glutamine, nonessential amino acids, penicillin, streptomycin, and 10% FCS. WEHI 231 B lymphoma and L cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 with L-glutamine, 50 μM 2-ME, 10% FCS, and antibiotics. The WB T lymphoma, kindly provided by Dr. Jayne Lesley (The Salk Institute, San Diego, CA), was used to derive the WB-N subclone. It was maintained in DMEM with 2-ME, L-glutamine, 10% FCS, and antibiotics. The IL7–dependent D6 pre–B cell clone has not been previously described. It was derived from the bone marrow of a bcl-2 transgenic mouse and maintained in supplemented RPMI 1640 medium with IL-7. The KM114 (IgG1) and KM81 (IgG2a) mAbs recognize both murine and hamster CD44 (14a). The IRAWB14 antibody to CD44, which enhances ligand binding (7), was originally donated by Dr. Jane Lesley. The KM16 (IgG2a) mAb (26) to β_1 integrin was used as an irrelevant isotype-matched control. Antibodies used for three-color flow cytometry analysis included 14.8-PE (CD45R) obtained from Pharmingen (San Diego, CA), S7 (CD43), which was purified and biotinylated in our lab from the hybridoma obtained from American Type Culture Collection, the 1126C-1 antibody to IgD prepared as described (27), rat anti–mouse IgM-FITC (Zymed Laboratories, Inc., South San Francisco, CA), rat anti–mouse CD44–FITC (Southern Biotechnology, Inc., Birmingham, AL), and biotinylated rat anti–mouse CD3 (GIBCO BRL, Gaithersburg, MD). These were detected with Cy-chrome-labeled streptavidin (Pharmingen) or FITC-streptavidin (Zymed Laboratories, Inc.).

Reagents. N'ase (from *Arthrobacter ureafaciens*) and a mixture of endoglycosidase F (endo-F) and *N*-glycosidase F (PNGase-F) (from *Flavobacterium meningosepticum*) were purchased from Sigma Chemical Co. (St. Louis, MO). Tunicamycin was from Calbiochem-Novabiochem Corp. (La Jolla, CA). FITC-conjugated *Maacia amurensis* lectin (MAA) and *Sambucus nigra* lectin (SNA) were from E-Y Laboratories (San Mateo, CA). SNA staining always diminished with N'ase treatment of cells and was used to confirm sialic acid release. MAA is actually a mixture of two lectins, which differentially recognize complex glycoconjugates (28). While its binding to cells decreased with tunicamycin treatment, it reproducibly increased after N'ase treatment (see Table 2). FITC-conjugated *Ulex europaeus* (UEA1), Con A, *Ricinus communis* (RCA_{120}), and wheat germ agglutinin (WGA) were purchased from Vector Laboratories, Inc. (Burlingame, CA).

Flow Cytometry. Cells were stained with fluorescein-conjugated HA (FL-HA; 3.5 μg/ml) (7) or FITC-conjugated SNA (20 μg/ml), MAA (20 μg/ml), RCA_{120} (20 μg/ml), UEA1 (20 μg/ml), Con A (20 μg/ml), or WGA (10 μg/ml) in FCS/PBS (PBS containing 5% FCS and 0.1% NaN_3) for 15 min at 4°C. Cells were washed with FCS/PBS and analyzed with a FACScan® (Becton Dickinson and Co., Mountain View, CA). Dead cells were gated out on the basis of propidium iodide staining. The CD44 dependence of FL-HA staining was always determined by blocking with the CD44-specific mAb KM114 (14a, 29). PE-labeled CD45R and either biotinylated CD43 or biotinylated anti-IgD mAb were used with FL-HA, followed by Cy-chrome-labeled streptavidin for three-color analyses. Cell sorting was carried out on a FACStar^{PLUS}® (Becton Dickinson and Co.) using wild-type CHO cells stained with FL-HA. The 5% most positive, or least positive, cells were sorted and then cloned by limiting dilution to yield the CHO/SKP and CHO/SKN subclones, respectively.

Preparation of sCD44–Ig. A soluble fusion protein was prepared containing the extracellular domain of murine hemopoietic CD44 and the hinge, CH2, and CH3 regions of human IgG_1 (CD44–Ig) as described previously by others (30). The Ig cassette was generously provided by Dr. A. Aruffo (Bristol-Myers, Evansville, IN). The CD44–Ig construct was prepared in the pEF-BOS vector and transfected into 293T human renal cell carcinoma cells with a standard calcium phosphate method (31). Supernatants were harvested after 5 d. CD44–Ig was then purified from the culture supernatants with a column of immobilized protein A (Pierce, Rockford, IL), and eluted protein was dialyzed against PBS. The concentration of purified CD44–Ig was determined by a CD44-specific ELISA as previously described (4).

Binding of HA to Immobilized CD44–Ig. Dilutions of CD44–Ig were agitated with protein A–coated agarose beads (Pierce) for 2 h at room temperature. After washing, the coated beads were treated with N'ase (1 U/ml) in 50 mM sodium phosphate, pH 6.5, overnight at 37°C. Controls were treated under identical conditions except without N'ase. The CD44–Ig–coated beads were then incubated with FL-HA for 30 min at 4°C. In some experiments, the beads were first incubated with anti-CD44 mAb (10 μg) for 15 min at 4°C and then stained with FL-HA. After a final wash, the beads were analyzed with a 400-μm nozzle on a FACStar^{PLUS}®

flow cytometer. N'ase treatment of uncoated protein A beads did not increase their background binding to FL-HA.

Immunoprecipitation and Immunoblotting. Cells were surface labeled with a biotinylation procedure (27). Live cells (10^7/ml) were incubated with sulfosuccimidobiotin (Pierce) (0.1 mg/ml) in labeling buffer (150 μM NaCl, 0.1 M Hepes, pH 8.0) for 30 min at room temperature. Cells were then washed with RPMI 1640 medium containing 3% FCS and lysed with 1% Triton X-100 in 10 mM Tris, pH 7.5, 150 mM NaCl, 3 mM EDTA, 50 mM iodoacetamide, 0.1% NaN_3, 1 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin, and 1 U/ml aprotinin for 1 h at 4°C. The detergent-solubilized materials were recovered after centrifugation and precleared with 50 μl of goat anti-rat IgG Sepharose 4B beads (Zymed Laboratories, Inc.) conjugated with irrelevant control KM16 (IgG_{2a}) mAb, followed by immunoprecipitation with either KM81 (IgG_{2a}) or KM16 as a negative control. After extensive washing, proteins were eluted from beads by boiling in nonreducing SDS sample buffer and analyzed by SDS-PAGE with a 7.5% gel, followed by transfer to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 0.5% gelatin in PBS plus 0.05% Tween 20 and 0.05% thimerosal for 1 h at room temperature, and it was incubated with 1/5,000 diluted streptavidin–horseradish peroxidase in PBS containing 1% BSA and 0.05% Tween 20. The protein bands were detected using the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL). Prestained molecular weight markers (Sigma Chemical Co.) were used as standards. CD44–Ig on the protein A beads was untreated or treated with N'ase and then analyzed by SDS-PAGE under reducing conditions. After transfer to the membrane, CD44–Ig was detected with 1/2,000 diluted horseradish peroxidase–coupled goat anti–human IgG (Southern Biotechnology, Inc.) and the enhanced chemiluminescence system.

Preparation and Culture of Splenic B Cells. B cells were prepared by treating spleen cells from 8–12-wk-old BALB/c or (NZB × NZW) F_1 mice with anti-Thy1.2 mAb plus complement (Accurate Chemical Corp., Westbury, NY) as previously described (32). B cells from NZB/W F_1 mice were mainly used because they are more sensitive to IL-5 than are those of BALB/c mice (33). The B cells (5×10^6/ml) were cultured for 3 d in RPMI 1640 supplemented with 50 μM 2-ME, penicillin, streptomycin, and 10% FCS. Stimulants were LPS (10 μg/ml) or IL-5 (1,000 U/ml) (a generous gift of Dr. Kiyoshi Takatsu, Tokyo University, Tokyo, Japan). After culture, >99% of cells were IgM positive.

Enzyme Treatment. Cells (2×10^6) were cultured in medium with addition of tunicamycin (10 μg/ml) overnight. They were then suspended (2×10^6/ml) in buffered medium (RPMI 1640/PBS, 1:1, pH 6.8) with N'ase (0.4 U/ml) or endo-F/PNGase-F (4 U/ml) and incubated for 2 h at 37°C with gentle shaking as reported by others (22). The reaction was terminated by washing. None of the enzyme treatments influenced cell viability. Control cells were treated under identical conditions, except without enzyme.

Statistical Analysis. The significance of differences was evaluated by means of paired Student's *t* tests using the SigmaStat program (Jandel Scientific, San Rafael, CA).

Results

HA Recognition by CHO Mutants and Sublines. CHO cell mutants and sublines have long been exploited for study of protein glycosylation (34–36), and we hoped they would be informative about CD44-mediated recognition of HA. In preliminary experiments, we screened a number of them and found variations in their ability to bind FL-HA. Strong CD44-mediated recognition of HA was observed with mutant Lec 8 cells, but there was heterogeneity of binding with wild-type CHO cells (Fig. 1 and data not shown). The latter were sorted and cloned on the basis of HA recognition, yielding two subclones (SKP and SKN), which differed for this property but had similar amounts of cell surface CD44 (Fig. 1). Fluorescent lectins were used to explore the possibility that the subclones might differ with respect to glycosylation (Table 1 and data not shown). The most significant difference was with the RCA lectin, which gave an average of 5.8-fold higher staining with the HA-nonbinding CHO/ SKN subclone than with the HA-binding CHO/SKP subclone ($P = 0.0001$) in eight independent experiments. Significant differences of less magnitude were obtained with WGA and MAA lectin ($P = 0.0002$ and 0.0004, respectively). The two subclones were similar with respect to staining by fluorescent Con A ($P = 0.578$). Lectins also confirmed that the Lec 8 cells were defective in glycosylation, as this mutant exhibited virtually no staining with either MAA or RCA (2 and 8% of wild-type values, respectively).

Lec 8 cells have been well characterized and are known to express incompletely glycosylated proteins (37). There is an inability to transport uridine diphosphate galactose from the

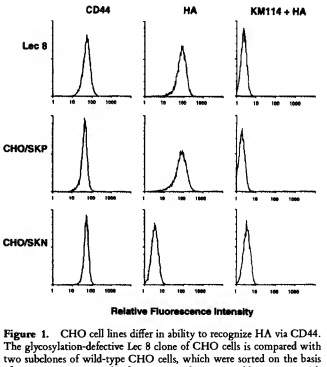

Figure 1. CHO cell lines differ in ability to recognize HA via CD44. The glycosylation-defective Lec 8 clone of CHO cells is compared with two subclones of wild-type CHO cells, which were sorted on the basis of HA recognition. Levels of CD44 were demonstrated by staining with biotinylated anti-CD44 mAb (KM114) followed by FITC-labeled streptavidin. Ligand binding was analyzed by exposure of cells to FL-HA. To investigate CD44 dependence of HA recognition, cells were preincubated with unlabeled KM114 before staining with FL-HA. All samples were then analyzed by flow cytometry.

Table 1. *Fluorescent Lectins Reveal Apparent Glycosylation Differences in CHO Cell Subclones*

Lectin	CHO/SKN	CHO/SKP
UEA1	5 ± 1	4 ± 1
Con A	35 ± 20	40 ± 29
RCA_{120}	619 ± 196	107 ± 39*
WGA	1489 ± 377	1104 ± 384*
MAA	214 ± 62	127 ± 34*

Cells were stained with FITC-labeled UEA1, Con A, RCA_{120}, WGA, or MAA and analyzed by flow cytometry. The values represent mean ± SD median fluorescence intensities of eight independent experiments. * Highly significant differences (RCA_{120}, $P = 0.0001$; WGA, $P = 0.0002$; MAA, $P = 0.0004$).

cytosol to the Golgi, resulting in proteins lacking galactose. The majority of sialic acids on cell surface glycoconjugates are linked to galactose, and, consequently, Lec 8 cells have markedly reduced cell surface sialic acid. Our findings demonstrate that expression of such mature, complex sugars is not required for CD44-mediated recognition of HA (Fig. 1). Furthermore, subclones of wild-type CHO cells, which differ with respect to HA binding, also differ in staining by three lectins. These findings indicated that certain protein glycosylation patterns may negatively influence the ability of cells to recognize HA via CD44.

Reductions in Glycosylation Increase HA Recognition by CHO Cells. The wild-type subclone of CHO cells that failed to recognize HA (CHO/SKN) was then exposed to tunicamycin overnight to disrupt N-linked glycosylation (38) under treatment conditions that had no influence on cell viability. Decreased staining with fluorescent RCA confirmed that glycosylation was affected. The density of cell surface CD44 as detected with the KM114 antibody was reduced on average by 32% (median fluorescence intensity, Fig. 2). Similar results were obtained with the KM81 antibody, which has similar specificity to KM114 (14a). Reduced transport of protein to the cell surface and/or partial loss of an epitope may account for the reduced staining with this antibody. Indeed, recognition of hamster cells by the KM201 antibody to murine CD44 was virtually abolished by tunicamycin treatment (data not shown).

In contrast to the apparent decrease in cell surface CD44, there was a dramatic increase in the ability of treated CHO/SKN cells to recognize FL-HA. In three experiments, treated cells bound ligand an average of 6.8-fold better than control cells. This binding was CD44 mediated and completely blocked by the KM114 mAb (Fig. 2).

Cell surface labeling and immunoprecipitation were then used to evaluate the glycosylation status of CD44 on CHO cell subclones and treated cells. The principal species of CD44 in Lec 8 cells was ~70 kD in size and clearly smaller than the corresponding bands in wild-type subclones (Fig. 3 *A*). A small, but consistent, difference in CD44 size was found between the two subclones. We estimate that CD44 immunoprecipitated from the HA-binding subclone (CHO/SKP) was ~4 kD smaller than that obtained from the HA-nonbinding subclone (CHO/SKN). Tunicamycin treatment always generated a very small (~58 kD) species of cell surface CD44 (Fig. 3 *B*), and, in some experiments, virtually all of the CD44 was converted to this reduced size.

Enzyme treatments were then used to evaluate the relationship between protein glycosylation and HA recognition. In two experiments, treatment of the non-HA-binding subclone (CHO/SKN) with the combination of endo-F and PNGase-F significantly increased ligand recognition (Fig. 4). An even more dramatic increase followed treatment of cells with *A. ureafaciens* N'ase. Neither of these treatments influenced cell viability, and HA recognition was completely CD44 mediated (data not shown). Furthermore, the influence of these enzymes on cell surface glycoproteins was confirmed by staining with fluorescent lectins RCA and MAA (Fig. 4). Staining with RCA reproducibly decreased while that with MAA increased. The latter is actually a mixture of two lectins

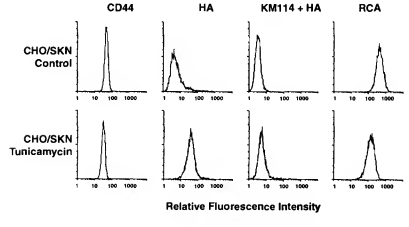

Figure 2. Tunicamycin treatment induces HA recognition on a nonbinding subclone of CHO cells. Cells were incubated with or without tunicamycin (10 μg/ml) overnight at 37°C, which resulted in a slight loss of CD44 as detected with biotinylated KM114. Ligand binding was assessed with FL-HA and CD44 specificity by preincubation with unlabeled KM114 antibody. The FITC-labeled RCA lectin was used to assess changes in cell surface glycoproteins. All cells were analyzed by flow cytometry.

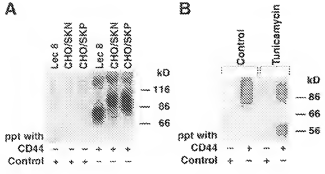

Figure 3. Sizes of CD44 on CHO cell subclones. (*A*) Cells were surface labeled with biotin and lysed with Triton X-100. Extracts were precleared and immunoprecipitated with anti-CD44 mAb (KM81) or control mAb (KM16). (*B*) HA-nonbinding CHO cells (CHO/SKN) were cultured with or without tunicamycin (10 μg/ml) overnight at 37°C and surface labeled with biotin. Triton X-100 extracts were immunoprecipitated as described above. Samples were analyzed by SDS-PAGE (7.5%) under nonreducing conditions and visualized as described in Materials and Methods.

with complex specificity for glycoproteins (28), and we assume that cryptic binding sites were exposed by release of terminal sialic acids. All of these observations would be consistent with a negative regulatory role for glycosylation on CD44-mediated HA recognition in CHO cells.

N'ase Treatment Increases HA Recognition by Particular Lymphoma Cells. Previous studies revealed that lymphoid cells can be divided into categories with respect to HA recognition (2). One type of cells, typified by resting splenic B cells, or an IL-7–dependent pre–B cell clone (D6), does not bind HA and is unaffected by the enhancing IRAWB14 mAb. We found that neither N'ase treatment of either cell type nor tunicamycin treatment of the pre–B cell line influenced the ability of these cells to bind HA (Table 2). Staining with fluorescent lectins confirmed that glycoproteins were modified by these manipulations. Investigation of possible effects of tunicamycin on nonstimulated spleen cells was not possible because of poor cell viability. We also tested a fibroblast cell line (L cells), which has only partial responsiveness to the IRAWB14 antibody (results not shown), and we found that

Table 2. *Effect of Deglycosylation on HA Recognition*

Cells	Treatment	Median fluorescence intensity: CD44	HA	KM114 + HA
Splenic B	Control	52	5	5
	N'ase	60	9	10
D6	Control	81	5	6
	Tunicamycin	54	3	3
	Control	70	5	6
	N'ase	96	5	6
L cell	Control	60	2	2
	Tunicamycin	20	7	3
	Control	87	2	2
	N'ase	78	7	2
WB-N	Control	109	11	6
	Tunicamycin	116	46	5
	Control	111	15	4
	N'ase	129	41	5
W231	Control	149	10	4
	Tunicamycin	75	31	4
	Control	107	15	2
	N'ase	104	30	2

Cells were treated with tunicamycin or N'ase and stained with FL-HA, SNA, MAA, or biotinylated KM114 plus FITC-streptavidin before flow cytometry analysis. As a specificity control, cells were stained with FL-HA after incubation with KM114. The values represent median fluorescent intensities after subtraction of background fluorescence. Representative results were from one of two to three similar experiments. The first three cell types failed to bind HA in the presence of IRAWB14, but the other two cell lines were able to bind HA after incubation with IRAWB14.

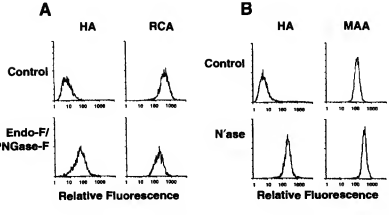

Figure 4. Enzymatic deglycosylation increases HA recognition by CHO cells. HA-nonbinding CHO cells (CHO/SKN) were treated with endo-F/PNGase-F or N'ase as described in Materials and Methods. They were then stained with FL-labeled ligand (HA) or either FITC-labeled RCA or MAA lectins before being analyzed by flow cytometry.

they were very slightly influenced by treatment with tunicamycin or N'ase (Table 2).

Another category of cells, typified by certain lymphomas and resting T lymphocytes, does not constitutively bind HA but does so in the presence of the enhancing IRAWB 14 antibody (2, 7). The WEHI 231 B lymphoma and the WB-N T lymphoma line have these properties, and we now show that they are similar to CHO cells in that they are responsive to deglycosylation (Table 2). This was true even in experiments in which tunicamycin treatment markedly reduced cell surface CD44 density (Table 2). All examples of HA binding were CD44 mediated and completely sensitive to the blocking KM114 antibody. These results reveal that glycosylation may represent one mechanism for negative regulation of HA recognition by certain CD44-bearing cells. However, there must be additional mechanisms for controlling this process, because some CD44-bearing cells remained unable to bind ligand under the same treatment conditions.

Characteristics of Activated B Cells That Can Recognize HA. While normal resting B cells do not bind HA, activation that accompanies mild graft-versus-host disease or exposure to IL-5 produces a small subpopulation of lymphocytes that has this capability (5, 39, 40). Several days are required for this change, which correlates with a subtle change in glycosylation of CD44 (40). We have now extended those findings by use of three-color flow cytometry, which revealed that HA-binding B cells were induced to express the CD43 antigen (Fig. 5, *A* and *B*, depicts LPS- or IL-5–activated B cells, respectively). All cells with high levels of CD44 were also positive for CD43. A selective loss of IgD represents an additional property of HA-binding B cells (Fig. 5). The transition of resting B cells (small in size, IgM^+, IgD^+, $CD43^-$, $CD44^{lo}$) that did not recognize HA to fully activated cells (large in size, IgM^+, IgD^-, $CD43^+$, $CD44^{hi}$) corresponded to the appearance of HA-binding lymphocytes. This was true regardless of whether LPS (Fig. 5 *A*) or IL-5 (Fig. 5 *B*) was used as an activation stimulus. However, HA-binding lymphocytes still only represented a subpopulation of activated B cells. These studies demonstrate that cell surface markers can be used to identify activated lymphocyte populations that include those that have acquired the ability to bind HA. A fraction of activated cells with similar surface characteristics still did not recognize this ligand.

A Subset of Activated B Cells May Be in a Transitional State. We then explored the possibility that glycosylation contributes to the inability of some partially activated B cells to recognize HA. Indeed, when splenic B cells were activated with either LPS or IL-5, twice as many bound HA after an additional treatment with N'ase (Table 3). This increase in numbers of HA-binding cells was not accompanied by an increase in the amount of ligand bound per cell, and there was no influence of treatment on CD44 density (as reflected by median channel numbers). Again, all HA recognition was CD44 mediated. We conclude that the glycosylation status of some cell surface protein(s) on a subset of activated B lymphocytes negatively influences their ability to recognize HA. This contrasts with resting B cells that were not induced to bind ligand by this single treatment (Table 1).

Removal of Sialic Acid Increases HA Recognition by a CD44–Ig Fusion Protein. Multiple cell surface proteins are influenced by treatments that inhibit or reverse glycosylation events, and it was unclear whether carbohydrate modification of CD44, or some other protein, influenced its ligand-binding ability. Therefore, we prepared a soluble fusion protein consisting of the extracellular portion of murine hemopoietic CD44 with the hinge, CH2, and CH3 domains of human IgG_1. This approach has been extensively used by others to investigate the intrinsic HA recognition potential of human CD44 (15, 30, 41). Graded amounts of the purified fusion protein were adsorbed to protein A–conjugated Sepharose beads, which were washed before exposure to a constant amount of fluorescent HA (Fig. 6 *A*). The resulting binding, as assessed by flow cytometry, was CD44 specific and completely blocked by treatment with the monoclonal KM114 antibody (Table 4). Pretreatment of the CD44–Ig–coated beads with N'ase increased their ability to recognize HA up to sevenfold, while enzymatic treatment of uncoated control beads did not result in binding. The enhancing effect of N'ase treatment on

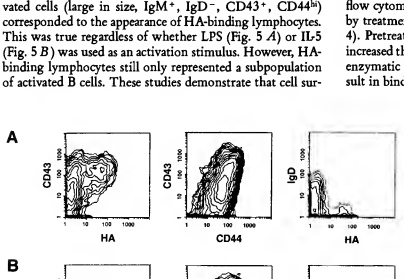

Figure 5. Characteristics of HA-binding B cells after activation in culture. T cell–depleted splenic B cells from NZB/W F_1 mice were cultured with LPS (10 μg/ml) (*A*) or IL-5 (1,000 U/ml) (*B*) for 3 d before analysis by three-color flow cytometry. All contour plots were obtained by gating on $CD45R^+$ cells. The left panels demonstrate ligand (HA)-binding cells and their expression of the CD43 antigen. The middle panel analyzes dual expression of CD43 and CD44 antigens. The right panel demonstrates that all HA-binding cells have lost the IgD isotype of Ig; however, they were still IgM positive (not shown). These results are typical of those obtained in three independent experiments.

Table 3. *Effect of Deglycosylation on HA Recognition by Activated Splenic B Cells*

Treatment	HA		CD44	KM114 + HA	SNA
	% *Positive*	*MFI*	*MFI*	*MFI*	*MFI*
LPS	7.6 ± 0.7	26 ± 3	51 ± 5	1 ± 0	52 ± 13
LPS + N'ase	18.1 ± 1.2	30 ± 3	57 ± 3	1 ± 0	9 ± 2
IL-5	5.9 ± 1.2	52 ± 12	38 ± 1	1 ± 0	35 ± 6
IL-5 + N'ase	12.1 ± 2.8	46 ± 7	38 ± 3	1 ± 0	6 ± 1

T cell–depleted splenic cells were cultured with LPS (10 μg/ml) or IL-5 (1,000 U/ml) and treated with N'ase as described in Materials and Methods. The cells were then tested for HA binding by flow cytometry after staining with FL-HA. As a specificity control, cells were also incubated with the blocking antibody, KM114, followed by staining with FITC-HA. The values represent means ± SD percent HA-binding cells and/or median fluorescence intensities (*MFI*).

HA recognition was similar in three separate experiments and where fluorescent HA was used over a 100-fold concentration range (average of 3.6-fold increase). We recovered the CD44–Ig from control and N'ase beads and analyzed it by SDS-PAGE under reducing conditions (Fig. 6 *B*). Enzymatic treatment reduced the size and heterogeneity of the fusion protein.

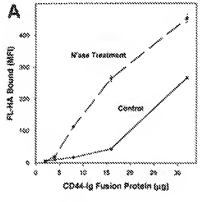

Figure 6. Desialylation of a CD44 containing fusion protein increases HA recognition. (*A*) Protein A–bearing beads were loaded with graded amounts of CD44–Ig, and 1 aliquot of each sample was treated with N'ase. The beads were then tested for HA-binding ability by flow cytometry. The results are shown as means ± SD. *MFI*, median fluorescence intensity. (*B*) CD44 was then extracted from similarly treated beads by boiling in SDS-PAGE buffer under reducing conditions. This material was analyzed by SDS-PAGE and Western blotting. The mobility of size standards is indicated.

The IRAWB14 antibody has been shown to markedly increase HA recognition on particular types of lymphocytes (2, 7, 14). We now show that it also has this effect on a CD44–Ig fusion protein immobilized on beads, regardless of whether the protein had been N'ase treated (Table 4). We conclude from these experiments that terminal sialic acids diminish the intrinsic HA-recognizing capability of CD44. The strong enhancing effect of the IRAWB14 antibody suggests that conformational changes in CD44 may also influence its function.

Discussion

CD44 is subject to enormous molecular diversity. We now show that changes in glycosylation status can markedly influence ligand recognition. Treatments that altered glycosylation in whole cells or purified CD44 significantly increased binding to HA. There is precedent for these findings with other cell adhesion molecules. This might be regarded as but one relatively slow mechanism through which cells can regulate adhesiveness.

Lymphoma subclones have been described that differ with respect to HA binding but are similar in terms of the number

Table 4. *CD44-specific Antibodies Influence HA Recognition by a CD44–Ig Fusion Protein Immobilized on Beads*

Antibody treatment	Untreated beads	N'ase-treated beads
Control	113	493
KM114	7	7
IRAWB14	3,162	2,839

CD44–Ig–coated beads were treated with N'ase and stained with FL-HA after incubation with anti-CD44 mAbs, KM114, or IRAWB14 (10 μg) for 15 min at 4°C. HA-binding ability was evaluated by flow cytometry. Values represent median fluorescence intensities from one of two similar experiments.

and sizes of CD44 molecules (2, 18). It has been suggested that local densities of CD44 on the membrane and/or conformational changes might partially explain differences in HA recognition between such cells (8, 14). Either type of change in CD44 would be regulated through interaction with other molecules, and there is evidence that both the transmembrane and cytoplasmic domains of CD44 are important (7, 8). Furthermore, palmitoylation or reversible phosphorylation of the molecule might result in dynamic associations with other molecules (9, 11–13, 42).

Heterogeneity in the structure of CD44 might also determine its ability to recognize HA. It has been demonstrated that one "epithelial" isoform of human CD44, which includes an exon product not commonly used by blood cells, had greatly reduced HA-binding ability (15). However, this might depend on the type of cell that expresses this alternately spliced CD44 isoform, because the murine homologue and even the human isoform bind HA in other circumstances (16, 17). As another example of structure/function heterogeneity, addition of chondroitin sulfate and heparan sulfate to the protein confers the ability to recognize the additional ligands collagen and fibronectin (43).

The core protein of CD44 is predicted to be only 42 kD, but it has at least five motifs for asparagine-linked carbohydrate attachment and 10 possible sites for O-linked glycosylation (44). The mature protein on most blood cells is ~90 kD, and the acidic charge (isoelectric point = 4.2) is largely contributed by sialic acids (45, and for review see reference 2). A number of studies have used enzymatic treatments to conclude that multiple carbohydrate modifications are differentially used by various cell types to diversify the structure of this protein (45–48). Puré and co-workers demonstrated that the CD44 on resting and activated macrophages differed not only with respect to phosphorylation and cytoskeletal association, but also in the degree of N-glycosylation (42). More recently, the CD44 on B lymphocytes was found to be slightly smaller after IL-5 activation, and the difference was lost after PNGase-F treatment (40). Carbohydrate side chains on CD44 can be recognized as blood group antigens, and it has been speculated that fucosylation might modulate functions of the molecule associated with the tumorigenicity of colon carcinoma cells (49).

Bourguignon and co-workers (47) reported that the smallest biosynthetic precursor of CD44 was unable to bind HA and that this function was acquired with addition of O- and N-linked sugars. We found that CD44 on the Lec 8 mutant of CHO cells avidly bound HA (Fig. 1). These cells are unable to transport uridine diphosphate galactose to the Golgi, resulting in incompletely glycosylated proteins (37). We confirmed that the major CD44 was of reduced size (Fig. 3). Therefore, while the HA-binding function might be acquired simultaneously with a minimum amount of carbohydrate during biosynthesis, complete processing is not required.

It will be important to determine why the ligand recognition function of CD44 is increased after N'ase treatment. The overall charge of the glycoprotein could be important, and sialic acids might interfere with cooperation between clustered molecules. Alternatively, carbohydrate might contribute in a positive or negative way to the actual ligand-binding site. In this context, it is interesting that the cross-reactivity of the KM201 antibody for hamster cells was completely lost by treatment with tunicamycin. This reagent has been used to block HA recognition by a variety of cell types in mice and humans (14a, 29, 50). Its epitope might therefore be close to the HA-binding region.

Carbohydrates constitute important ligands for members of the selectin family of cell adhesion molecules (36, 51–53). CD22 and sialoadhesin are two recently described proteins whose ligand specificity is sialic acid dependent (21). In addition, there are examples where the functions of integrins and Ig superfamily members may be influenced by glycosylation. N-linked glycosylation is reportedly essential for association of the α_5/β_1 integrin subunit chains as well as for recognition of the ligand fibronectin (22). On the other hand, N-linked side chains of intracellular adhesion molecule 1 (CD54) diminish its recognition by the CD11b/CD18 integrin (23). Furthermore, extensive sialylation of neural cell adhesion molecule acts as a negative regulator of adhesion, possibly by reducing homophilic recognition (24).

While integrin-dependent adhesiveness of cells can change rapidly (6, 54, 55), days are required to observe changes in the ability of stimulated lymphocytes to bind HA (5, 56). It has previously been demonstrated that either IL-5 stimulation or a graft-versus-host response generates a subpopulation of B lymphocytes that can bind HA (5, 39, 40). There was a corresponding increase in the size of the lymphocytes, as well as in the average density of CD44 and IgM, while CD45R expression decreased. As noted above, the degree of N-linked glycosylation of CD44 was also slightly less on activated cells (40). We now demonstrate two additional changes and show that LPS stimulation gives similar results to those that occur with IL-5 stimulation. HA-binding lymphocytes appeared in culture by 3 d after IL-5 or LPS addition. All of them had lost IgD and acquired CD43 (as recognized by the S7 mAb, Fig. 5). The latter marker is interesting in that it may share with CD44 the ability to associate with the ERM family of cytoskeletal proteins (10, 57). Like CD44, it is shed from the surface of activated cells, and it loses some sialic acid when lymphocytes become activated (58, 59). Furthermore, it has been reported that CD43 might interfere with interactions between the CD11b/CD18 integrin and intracellular adhesion molecule 1 (60). Moreover, N'ase treatment diminished the CD43 antiadhesive effect. We know of no evidence for physical association or functional cooperation between CD43 and CD44. It remains to be determined if it is mere coincidence that CD43 is acquired on cells that become able to use CD44. Among activated B cells, two subsets of approximately equal number were recognized. One constitutively bound HA, and the other did so after N'ase treatment.

The glycosylation status of CD44 could be important for regulating its roles in a wide range of biological processes (2). N'ase treatment of endothelial cells decreases lymphocyte attachment, while treatment of lymphocytes can increase the same interactions (61, 62). These observations were in part attributable to selectin ligands, but they demonstrate the

importance of glycoconjugates to lymphocyte migration patterns. Interestingly, terminal sialic acid residues on glycoproteins may inhibit the ability of resting B lymphocytes to conjugate with T cells, present antigens, and function as stimulators in mixed lymphocyte responses (63). Furthermore, it has been suggested that endogenous sialidase activity may contribute to the ability of activated B cells to effectively interact with T lymphocytes (64). Terminal sialic acids on CD44 diminish its HA recognition function, and it will be important to learn if this influences its interaction with other possible ligands (43, 65).

We found that disrupting glycosylation in resting B cells or pre-B cells was not sufficient to cause HA recognition, suggesting that additional regulatory mechanisms might be involved. This point was explored in recent independent studies conducted by Lesley et al. (66), who also found that the glycosylation status of cells correlates with their ability to recognize HA via CD44. Their analysis of variant subclones demonstrated that multiple steps are required for cells with no HA-binding ability to become constitutively active for this function. Additionally, one of these steps might involve proteoglycan side chains, because treatment of inactive cells with β-D-xyloside or chondroitinase ABC to disrupt or remove glycosaminoglycan chains converted cells to a stage where they were responsive to the IRAWB14 antibody.

These observations raise a number of interesting questions. It will be important to determine precisely which carbohydrate modifications control cell interactions via CD44. Changes in CD44 structure might then be attributed to particular biosynthetic and/or deglycosylation enzymes, whose expression is linked to signals from the microenvironment.

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